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(54) Title: FLT4 RECEPTOR TYROSINE KINASE AND ITS USE IN DIAGNOSIS AND THERAPY

(57) Abstract

Anti-FLT4 antibodies, especially monoclonal anti-FLT4 antibodies, which are useful as a specific marker for endothelial cells of lymphatic vessels and HEVs, as a diagnostic tool for detecting changes in lymphatic tissue, especially in lymphatic vessels and HEVs in disease states, such as lymphangioma, metastatic lymph nodes and inflammatory, infectious and immunological disease.

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FLT4 RECEPTOR TYROSINE KINASE AND ITS USE IN DIAGNOSIS AND THERAPY

FIELD OF THE INVENTION

The present invention relates generally to receptor tyrosine kinases, nucleic acid probes and antibodies specifically recognizing such receptors, and the use of such probes and antibodies for identifying lymphatic vessels and high endothelial venules (HEV) in animal and human tissues and lymphatic endothelial cells in culture. More specifically the present invention is directed to FLT4, a receptor tyrosine kinase, to nucleic acid probes recognizing FLT4, to antibodies specific to FLT4 and to methods for identifying FLT4 expression in lymphatic vessels and ultimately diagnosing and treating disease states in animals and humans involving changes in lymphatic tissue, such as inflammatory, infectious and immunological diseases, metastatic lymph nodes and lymphangiomas.

BACKGROUND OF THE INVENTION

The physiology of the vascular system, embryonic vasculogenesis and angiogenesis, blood clotting, wound healing and reproduction, as well as several diseases, involve the vascular endothelium lining the blood vessels. The development of the vascular tree occurs through angiogenesis and, according to some theories, the formation of the lymphatic system starts shortly after arterial and venous development by sprouting from veins (1,2).

After the fetal period endothelial cells proliferate very slowly, except during angiogenesis associated with neovascularization. Growth factors stimulating angiogenesis excert their effects via specific endothelial cell surface receptor tyrosine kinases.

The protein product of the FLT4 receptor tyrosine kinase cDNA, cloned from a human erythroleukemia cell line is N-glycosylated and contains seven immunoglobulin-like loops in its extracellular domain. The cytoplasmic tyrosine kinase domain of FLT4 is about 80 % identical at the amino acid

level with the corresponding domains of FLT1 and KDR and about 60 % identical with the receptors for platelet-derived growth factor, colony stimulating factor-1, stem cell factor and the FLT3 receptor [3].

Although the biological function of FLT4 are as yet unknown, its restricted expression pattern indicated that its functions may involve the vascular endothelium. Our previous results revealed FLT4 mRNA expression in endothelial cells in developing vessels of several fetal organs as disclosed by Kalpainen et al., in *J. Exp. Med.* 178: 2077-2088, 1993. A comparison of FLT4, FLT1, and KDR/FLK-1 receptor mRNA signals showed overlapping, but distinct expression patterns in the tissues studied [4]. These data suggested that the receptor tyrosine kinases encoded by this gene family may have distinct functions in the regulation of the growth and/or differentiation of blood vessels.

A major function of the lymphatic system is to provide fluid return from tissues and transport many extravascular substances back to the blood. In addition, during the process of maturation, lymphocytes leave the blood, migrate through lymphoid organs and other tissues, and enter the lymphatic vessels, and return to the blood through the thoracic duct. Specialized venules, high endothelial venules, (HEVs) bind lymphocytes again and cause their extravasation into tissues. The lymphatic vessels and especially the lymph nodes thus play an important role in immunology and they are also sites of development of metastasis of different tumors.

Since the turn of the 20th century, three different theories concerning the embryonic origin of the lymphatic system have been presented. However, prior to the present invention, lymphatic vessels have been difficult to identify, because there are no specific markers available for them.

Lymphatic vessels are most commonly studied with the aid of lymphography. In lymphography, X-ray contrast medium is injected directly into a lymphatic vessel. That contrast medium is distributed along the efferent drainage vessels of the lymphatic system. The contrast medium is collected in lymph nodes, where it stays for up to half a year, during which time X-ray analyses allow the follow-up of lymph node size and architecture. This

diagnostics is especially important in cancer patients with metastases in the lymph nodes and in lymphatic malignancies, such as lymphoma

SUMMARY OF THE INVENTION

The present invention is directed to FLT4 peptides and other constructs and to the use of FLT4 as a specific marker for lymphatic endothelial cells.

The invention is also directed to nucleic acid probes and antibodies specifically recognizing FLT4, especially to monoclonal antibodies, and compostions containing such antibodies. Further disclosed in the present application is the use of such monoclonal antibodies for diagnostic purposes for detecting and measuring the amount of FLT4 receptors in tissues, especially in lymphatic tissues and in lymphatic endothelial cells.

In a preferred embodiment, the invention provides monoclonal antibodies specifically recognizing the FLT4 receptor. More specifically this invention provides a monoclonal antibody designated 9D9F9. The hybridoma cell line which produces monoclonal antibody 9D9F9 is deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) under the provisions of the Budapest Treaty (DSM accession number ACC2210).

Monoclonal antibodies labelled with a detectable marker are also provided. As used herein, the term detectable marker encompasses any detectable marker known to those skilled in the art. However, in a preferred embodiment of this invention, the detectable marker is selected from the group consisting of radioisotopes, florochromes, dyes, enzymes and biotin. For the purpose of this invention suitable radioisotopes include, but are not limited to ^{125}I and ^{131}I.

Monoclonal antibodies of the present invention may also be used in a method for detecting the presence of FLT4-receptors in a cell sample, comprising the steps of exposing a cell sample to a monoclonal antibody of the present invention and detecting the binding of said monoclonal antibody to FLT4-receptors.

Another aspect of the present invention thus relates to a method of determining the presence of FLT4-receptors in a cell sample, comprising the steps of:

(a) exposing a cell sample to a monoclonal antibody of the present invention;

(b) detecting the binding of said monoclonal antibody to FLT4-receptors.

The exposure of a cell mixture to monoclonal antibodies of the invention can be in solution, as is the case for fluorescence-activated cell sorting, or it can be on solid tissue specimens, such as biopsy material, or it can be with the monoclonal antibody immobilized on a solid support, as is the case with column chromatography or direct immune adherence. The mixture of cells that is to be exposed to the monoclonal antibody can be any solution of blood cells or tissue cells. Prefereably, the cell mixture is from normal or pathological tissue containing or suspected to contain lymphatic endothelial cells. After exposure of the cell mixture to the monoclonal antibody, those cells with FLT4 -receptors will bind to the monoclonal antibody to form an antibody-FLT4 -receptor complex. The presence of the antibody-FLT4 -receptor complex, and therefore FLT4 receptors, can be detected by methods known in the art. These methods include immunohistochemical methods standard in the art, such as immunofluorescence, FACS analysis, ELISA, IRMA (a sandwich type of immunochemistry assay), immunohisto-chemistry, RIA using ^{125}I-label and autoradiography.

The present invention also provides monoclonal antibodies conjugated to an imageable agent. As used herein, the term imageable agent includes, but is not limited to, radioisotopes. A preferred radioisotope is 99m-technetium.

In a specific embodiment, the invention is directed to a method for monitoring lymphatic vessels and their endothelial cells in tissue samples and in organisms. The present invention further provides clinical detection methods describing the state of lymphatic tissue, ans especially lymphatic vessels (inflammation, infection, traumas, growth, neoplasia etc.) and methods for detecting lymphatic vessels and thus lymphatic vascularization in an organism.

More specifically the present invention provides a method for detecting and identifying lymphatic changes charactherized by FLT4 expression in connection to metastatic cancers, inflammatory, infectious and immunological conditions, which method comprises the steps of

(a) obtaining a tissue and/or body fluid sample suspected of lymphatic changes, and

(b) contacting said sample with a FLT4-specific monoclonal antibody under conditions suitable for forming a complex between the monoclonal antibody and the antigen, and

(c) detecting the prescence of any complex formed.

A tissue which may be detected by this method is any normal, precancerous or cancerous solid tumor tissue with FLT4-containing lymphatic cells or cells which express the FLT4-receptor. In one embodiment of the present invention, the monoclonal antibody is labelled with a detectable marker as described herein. Methods of the invention are useful for detecting and differentiating various forms of cancer, especially metastases in the lymph nodes and other lymphatic malignancies, such as lymphomas, as well as lymphangiomas.

A method of imaging the presence of lymphatic vessels, high endothelial venules or lymph nodes in human patients, is also provided by this invention. This method comprises administration of labelled antibodies and detection by imaging at sites where FLT4 expressing cells are present, in lymphatic vessels or lymph nodes.

The invention is further directed to a method of stimulating or antagonizing the function of FTL4 in lymphatic vascularization and in inflammatory, infectious and immunological conditions, said method comprising inhibiting the FLT4-mediated lymphatic vascularization by providing amounts of a FLT4-binding compound sufficient to block the FLT4 endothelial cell sites participating is such reaction, especially where FLT4 function is associated with a disease such as metastatic cancers, lymphomas, inflammation (chronic or acute), infections and immunological diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Expression of FLT4 mRNA in mouse tissues A. Hybridization of polyadenylated RNA isolated from the indicated tissues of adult mice. The size of the FLT4 mRNA band is given in kilobases. B. RNAse protection analysis of RNA isolated from mouse embryos of various gestational ages (E8-E18) and from a newborn mouse (1 day). Sample E8+P contains also the placenta. The size of the probe and the protected FLT4 fragment are given in nt; ß-actin was used as a control.

Figure 2. Expression of FLT4 mRNA in a 7.5-, 8.5- and 11.5-day p.c. embryos. Darkfield and brightfield photomicrographs of *in situ* autoradiograms are shown. No expression of FLT4 mRNA could be detected in a 7.5-day embryo (A). The FLT4 expression of an 8.5-day p.c. mouse embryo is shown in (B) and (C). Arrows point to FLT4 positive cells in the endothelium of posterior cardinal vein (cv), in the allantois (al) in (B) and in angioblasts (ab) of the head mesenchyme in (C). In a 8,5-day p.c. placenta FLT4 transcripts can be seen in endothelial cells of venous lacunae (vl) (D). Panels E and F show a comparison of FLT4 and Tie hybridization signals in 11.5-day p.c. embryos. The region of the developing dorsal aorta and metanephros (mn) is shown (20x). Note that the dorsal aorta is negative for FLT4, but positive for Tie mRNA, whereas both probes hybridize with the endothelium of the subendocardial vein (sv). Also, the FLT4 probe gives a signal from the metanephric vein (v), whereas Tie mostly hybridizes with the metanephric capillaries (c, arrows). da: dorsal aorta, ng: neural groove. Scale bar: 30 µm.

Figure 3. FLT4 mRNA expression in a 12.5-day p.c. embryo. A sagittal section through the axillar plane is shown. Note that FLT4 mRNA is prominent in dilated vessels of the axilla (ax), in a plexus-like pattern in the periorbital (po) region, in the paravertebral tissue (arrowheads) and in the subcutaneous (sc) tissue. b: brain, li: liver. Scale bar: 5 µm.

Figure 4. FLT4 in 14 and 16.5 day p.c. embryos. Panels A and B show bright and darkfield images of a midsagittal section. po: periorbital region, lj: lower jaw, ne: neck region, sc: subcutis, mt, mesenterium, ao, aorta. dt: thoracic duct. (C) shows a transverse section of a 16.5 day embryo in

hematoxylin-eosin staining. th: thymus, tr: trachea, e: esophagus, ca: carotid artery, ba: brachiocephalic artery, dt: thoracic duct. (D) shows a higher magnification (40x) of the region of ductus thoracicus; the autoradiographic grains can be seen over the endothelial cells. Also, the small vessel (v) in the upper part of the photograph is positive. Scale bar: 10 µm (A-C), 1µm (D).

Figure 5. Comparison of FLT4 and Tie mRNA expression in cultured endothelial cells. Northern blot analysis of polyadenylated RNA from human foreskin microvascular (MV), femoral vein (VE), aortic (AO) and umbilical vein (HU) endothelial cells. For a comparison, the hybridization signal of the Tie receptor tyrosine kinase mRNA is shown. The bands resulting from the unspecific binding of the probe to the ribosomal RNA are marked with asterixes.

Figure 6. FLT4 in adult human lymphatic vessels of the mesenterium (A,B), lung (C,D), and tonsil (E,F). Note that only the lymphatic vessels in A and C give a FLT4 signal, whereas the veins, capillaries and the arteries are negative for FLT4 mRNA. In the tonsil, the signal is found in the endothelia of some HEVs. Scale bar: 200 µm.

Figure 7. FLT4 mRNA in normal (A, B) and metastatic (C, D) lymph node and in lymphangioma (E-G). Arrowheads mark the lymphatic sinuses and HEVs, which are FLT4 positive. A comparison of FLT4 and von Willebrand factor signals shows both in the lymphatic endothelium, but only von Willebrand factor signal in the capillary (c) and venous (v) endothelia. Scale bars: 10 µm (A-D) and 100 µm (E-G).

Figure 8. FLT4 expression in fetal mesenterial vessels detected by immunoperoxidase staining. Sections were stained with affinity-purified anti-FLT4 antibodies (A), with antigen-blocked antiserum (B) and with preimmune serum (C) and with antiserum specific against the factor VIII-related antigen (D). Note that staining is confined to some, but not all vessels (v). Scale bar, 0.05 mm.

DETAILED DESCRIPTION OF THE INVENTION

Recognizing the importance of identifying changes in lymphatic tissues, especially in lymph nodes in connection to metastatic cancers and immunological disease states, the present inventors have shown that FLT4 is a specific marker that detects lymphatic vessel endothelium and therefore useful as a marker for lymphatic changes in pathological states in man.

The present inventors have earlier shown that the expression pattern of FLT4 in comparison to FLT1 and KDR differs greatly in tissues of 18-week-old human fetuses [4]. In order to understand the role of FLT4 during development, the inventors cloned partial cDNAs for mouse FLT4. Using these probes in *in situ* hybridization, FLT4 mRNA expression during mouse development was analysed and it was found that FLT4 is expressed during vasculogenesis and angiogenesis of the lymphatic system. The relevance of these fingings was also confirmed in normal and pathological human adult tissues, as FLT4 was found in lymphatic endothelial cells of human adult tissues both in normal and pathological conditions, as well as in some high endothelial venules (HEVs).

The cloning of mouse FLT4 cDNA fragments showed that their deduced amino acid sequence is almost identical with the corresponding human sequence (amino acid identity about 96 % in both segments studied). Further evidence for the identity of the mouse FLT4 cDNA was obtained from Northern hybridization where probes from both species yielded the typical 5.8 kb mRNA signal from mouse tissues. Analysis of RNA isolated from various tissues of adult mice showed FLT4 expression in the liver, lung, heart, spleen and kidney, with no or very little hybridization in the brain and testes. This pattern is similar to the pattern reported earlier by Galland et al. [5]. The results of RNase protection suggested that the FLT4 gene is needed during mouse development, starting from 8.5 day p.c. embryos, and the relative expression levels appeared quite stable.

For the *in situ* hybridization two fragments of mouse FLT4 cDNA were selected, which encode sequences of the extracellular domain. This allowed a clear distinction of the hybridization pattern from the related FLK-1 and FLT-

1 receptor patterns, which show only a very low degree of sequence identity with FLT4 in the extracellular region [6, 7, 8, 9]. FLT4, similarly to the FLK-1, FLT-1, Tie and Tek endothelial receptor tyrosine kinase genes was not expressed in 7.5 day p.c. embryos. In a 8.5-day p.c. embryo the strongest FLT4 signals were localised in the allantois, the angioblasts of head mesenchyme and the cardinal vein. In contrast, the the dorsal aorta, endocardium of the heart and angioblasts of the yolk sac were negative, unlike for Tie, Tek, FLK-1 and FLT-1, Tie and Tek [10, 8]. The restriction of FLT4 expression to the venous system was even more clear in samples from 11.5 day mouse embryos; where the Tie mRNA was expressed also in arteries. In 12.5-day p.c. embryos the FLT4 signal decorated developing venous and presumptive lymphatic endothelia, but unlike for the endothelial Tie receptor tyrosine kinase, arterial endothelia were negative. During later stages of development FLT4 mRNA became restricted to vascular plexuses devoid of blood cells, representing developing lymphatic vessels. Only the lymphatic endothelium and some high endothelial venules expressed FLT4 mRNA in adult human tissues. Increased expression occurred in lymphatic sinuses and high endothelial venules in metastatic lymph nodes and in lymphangioma.

Due to difficulties in the interpretation of data from mouse embryos, human endothelia were studied, because the lymphatic system is much better defined in humans. Also, cells established from various endothelia could be studied in cell culture to see if the specificity of FLT4 expression persists in *in vitro* conditions. Endothelial cells lines are known to lose differentiated features upon in vitro culture. Therefore, it was not unexpected that they were negative for FLT4. Cultured aortic endothelial cells were also devoid of FLT4 mRNA. However, signals were obtained from human endothelial cells grown from the microvasculature and from femoral and umbilical veins. Thus, at least some of the specificity of FLT4 expression was retained in cell culture.

In situ hybridization analysis of adult human tissues confirmed the restriction of FLT4 to the lymphatic system seen in the developing mouse embryos. FLT4 expression was seen in the lymphatic endothelia and in the sinuses of human lymph nodes. Interestingly, also some of the HEVs, which have a cuboidal endothelium, shown to function in the trafficking of

leukocytes to the lymph nodes, were FLT4 positive. Furthermore, a parallel hybridization analysis showed that FLT4 mRNA levels were enhanced in these structures in metastatic as compared to normal lymph nodes. FLT4 was also very prominent in lymphangiomas, which are benign tumours composed of connective tissue stroma and growing, endothelial-lined lymphatic channels. FLT4 mRNA was restricted to the lymphatic endothelium of these tumors and absent from their arteries, veins and capillaries. In the human lung we were able to identify lymphatic structures, which were the only FLT4 positive vessels in this tissue.

The foregoing results suggest that FLT4 is a novel marker for lymphatic vessels and some high endothelial venules in human adult tissues. They also support the theory on the venous origin of lymphatic vessels. FLT4, as a growth factor receptor, may be involved in the differentiation and functions of these vessels.

These results, combined with the FLT4-binding compounds according to the present invention, allows a selective labelling of lymphatic endothelium, especially by using antibodies of the present invention coupled to radioactive, electron-dense or other reporter substances, which can be visualized. It may be possible to inject into the lymphatic system substances, containing FLT4 receptor internalization-inducing monoclonal antibodies, and thereby transport predefined molecules into the lymphatic endothelium. Also, it may be possible to use such the FLT4-binding compounds according to the invention for the detection of high endothelial venules, especially activated HEVs, which express enhanced levels of the FLT4 receptor. To our knowledge, no such specific markers are currently available for lymphatic endothelium.

The following examples are given merely to illustrate the present invention and not in any way to limit its scope.

EXAMPLES

EXAMPLE 1

Cloning of mouse FLT4 cDNA probes

Approximately 10^6 plaques from a lFIX®II genomic library from 129SV mice (Stratagene) was screened with the S2.5 human FLT4 receptor cDNA fragment covering the extracellular domain [3]. A 2.5 kb Bam HI fragment was subcloned from a positive plaque and sequenced from both ends. From this subclone, polymerase chain reaction was used to amplify and clone into the pBluescript KSII+/- vector (Stratagene) an exon fragment covering nucleotides 1745-2049 of the mouse FLT4 cDNA sequence [9].

A second fragment covering nucleotides 1-192 was similarly cloned.

EXAMPLE 2

Analysis of FLT4 mRNA in mouse tissues

Total RNA was isolated from developing embryos (8-18 days p.c. and one day old mice) according to Chomczynski et al. [11]. The sample from 8 day p.c. embryos included also the placenta.

For RNase protection analysis, RNA probe was generated from the linearized FLT4 plasmid obtained according to Example 1 using [^{32}P]-UTP and T7 polymerase for the antisense orientation. The ß-actin probe used corresponds to nucleotides 1188-1279 of the published mouse ß-actin sequence [12]. After purification in a 6% polyacrylamide/7M urea gel, the labelled transcripts were hybridzed to 30 µg of total RNA overnight at 52 °C. Unhybridized RNA was digested with RNase A (10 U/ml) and T1 (1 µg/ml) at 37 °C, pH 7.5 for 1 h. The RNases were inactivated by proteinase K digestion at 37 °C for 15 min and the samples were analysed in a 6% polyacrylamide/7M urea gel.

The pattern of expression of FLT4 analysed in this experiment showed that very weak mRNA signals were obtained from lung, liver, heart, kidney, skeletal muscle and spleen, whereas testis and brain were apparently

without specific signal (Fig. 1A). Analysis of a series of RNAs collected during different phases of mouse development by RNase protection assay showed that the FLT4 mRNA was expressed throughout embryogenesis from day 8 p.c. to newborn mice without great variations in signal intensity (Fig. 1B).

EXAMPLE 3

In situ hybridization for FLT4 in mouse embryos

To better assign FLT4 transcripts to cells and tissues, sections of 7.5 and 8.5 day p.c. mouse embryos were hybridized with labelled FLT4 RNAs. Mouse embryos were derived from matings of CBA and NMRI mice. Pregnant mice were killed by cervical dislocation and the embryos were either immediately frozen or transferred via phosphate buffered saline into 4% paraformaldehyde. The embryos and isolated mouse organs were fixed for 18 h at 4°C, dehydrated, embedded in paraffin and cut into 6 µm sections.

RNA probes (antisense and sense) of 192 and 349 nucleotides (see Example 1) were generated from linearized plasmids using [^{35}S]-UTP. *In situ* hybridization of sections was performed according to Wilkinson et al. [13, 14], incorporated by reference herein, with the following modifications: 1) instead of toluene, xylene was used before embedding in paraffin wax, 2) 6 µm sections were cut, placed on a layer of diethyl pyrocarbonate-treated water on the surface of glass slides pretreated with 2% 3-triethoxysilylpropylamine, 3) alkaline hydrolysis of the probes was omitted, and 4) the high stringency wash was for 80 min at 65°C in a solution containing 30 mM DTT and 1 x SSC. The sections were covered with NTB-2 emulsion (Kodak) and stored at 4°C. The slides were exposed for 14 days, developed and stained with hematoxylin. Control hybridizations with sense strand and RNase A-treated sections did not give a specific signal above background.

As shown in Figures 2A and B, FLT4 mRNA was not expressed in 7.5 day p.c. mouse embryos, but bright signals were detected in the posterior cardinal vein (cv) on day 8.5 of development. In contrast, the developing heart (data not shown) and dorsal aorta (da) were FLT4-negative. In the extraembryonic tissues FLT4 was prominently expressed in the allantois (al

in panel B), whereas developing blood islands of the yolk sac were negative (data not shown). On the other hand, angioblasts (ab) of the head mesenchyme were strongly FLT4 positive (C). In the developing placenta FLT4 signal was first seen in peripheral sinusoidal veins (data not shown). In 9.5 day p.c. placenta the endothelium of venous lacunae (vl in D) and the giant cells partially fused to the Reichert's membrane (data not shown) expressed FLT4 mRNA.

Thus, although FLT4 expression was very prominent in the earliest endothelial cell precursors, the angioblasts, it appeared to be restricted only to certain vessels of 8.5 day p.c. embryos. The Tie receptor is known to be expressed in all endothelial cells of developing mouse embryos and thus provides a marker for these cells. Notably, in contrast to the Tie probe, the FLT4 probe hybridized very weakly if at all with arterial endothelia of 11.5 day p.c. embryos, e.g. with the endothelium of the developing dorsal aorta (da in Fig. 2 E,F) or the carotic arteries (data not shown). Instead, FLT4 signal was much more prominent in the developing veins. For example, FLT4 signal was detected veins surrounding the developing metanephros (v, sv in E), while the Tie probe predominantly recognized capillaries (c) within the metanephros (F).

As can be seen from Figure 3, FLT4 mRNA is distributed in several regions of a 12.5 day p.c. mouse embryo, being particularly prominent in the dilated vessel of the axillar region (ax). A similar FLT4 positive vessel structure was seen in the mid-sagittal section in the jugular area (data not shown). A plexus-like pattern of FLT4 expressing vessels appeared in the periorbital region (po) and surrounding the developing vertebrae (vb). Also, just beneath the developing skin, a FLT4-positive vascular network was evident (sc). Weaker capillary signals were obtained from several regions, including the developing brain (b). FLT4 mRNA could also be detected in small vessels of the neck region, of the developing snout and at the base of the developing tongue as well as in the tail region (data not shown). Besides, the liver (li) was strongly positive for FLT4 mRNA in a spotlike pattern.

During further development, FLT4 RNA appeared to become more restricted to certain vessels of the embryo. A 14.5 day p.c. embryo shows

nicely this restricted pattern of expression (Fig. 4 A,B). In the midsagittal section of Figure 4 the most prominent FLT4 signal is seen along the developing vertebral column in its anterior part. This signal was considered to originate from endothelial cells of the thoracic duct (dt), which is the largest lymphatic vessel formed at this time of development. In contrast, the dorsal aorta (da) and inferior vena cava (vc) were negative. Dilated vessels in the mesenteric region were also strongly positive for FLT4. Furthermore, as in the 12.5 day p.c. embryos, vessel networks along anatomical boundaries in the periorbital (po), lower jaw (lj) as well as in the neck (ne) region contained FLT4 positive endothelia. Similar stuctures were present in the pericardial space and throughout the subcutaneous (sc) tissue. Notably, in contrast to FLT4 negative vessels, all FLT4 positive vessels were devoid of blood cells in their lumen. These expression patterns suggest that FLT4 becomes confined to the endothelia of lymphatic vessels at this time of development. An additional site where we observed FLT4 expression, was in the sinusoids of the developing bone marrow (bm).

Photographs of a transverse section of the upper thorax of a 16.5 day p.c. embryo hybridized with the FLT4 probe are shown in panels C and D of Figure 4. The section shown in C has been stained with hematoxylin-eosin to visualize the different types of vessels in this area. These include the carotic and brachiochepalic arteries (ca, ba), the vena cava (vc) and the thoracic duct, which is smaller in size and lacks surrounding muscular and connective tissue (arrow). A magnification of the region of thoracic duct is shown in panel D, where the FLT4 autoradiographic grains can be seen. Endothelial cells of the thoracic duct as well as a small vessel (v) in the vicinity hybridize with the FLT4 probe.

EXAMPLE 4

Analysis of FLT4 mRNA in cultured endothelial cells

The *in situ* hybridization results described in Example 3, showed that FLT4 is expressed in venous endothelial cells and later in lymphatic vessels and some venous endothelial cells, but not in arterial endothelia. In order to see if such regulation was maintained *in vitro*, we studied cultured endothelial cells using Northern blotting and hybridization analysis.

Endothelial cells from human aorta, femoral vein, umbilical vein, and from foreskin microvessels were isolated, cultured and characterized as previously described by Van Hinsberg, [15, 16]. They were used at confluent density after five to eight passages (split ratio 1:3) for the isolation of polyadenylated RNA.

The endothelial cell lines EA·hy926, BCE and LEII did not express FLT4 (data not shown). However, cultured human microvascular, venous and umbilical vein endothelial cells were positive for the FLT4-specific 5.8 and 4.5 kb mRNAs, whereas the aortic endothelial cells were negative (Fig. 5). In contrast, another endothelial receptor tyrosine kinase gene, Tie, was expressed as a 4.4 kb mRNA in all endothelial cell types studied.

EXAMPLE 5

FLT4 mRNA in in adult human tissues

The results obtained in Example 3 indicated that the FLT4 mRNA becomes largely confined to the endothelium of lymphatic vessels during development. Because of the potential significance of this finding in humans, we also studied FLT4 in adult human tissues using a human FLT4 probe. The human FLT4 probe used was an EcoRI-SphI fragment covering base pairs 1-595 of the cDNA [3]. The von Willebrand factor probe was an EcoRI-HindIII fragment covering base pairs 1-2334 [17].

We used routinely fixed material sent for histopathological diagnosis. Normal lung tissue was obtained from a resection of the left inferior lung lobe affected by epidermoid cancer. Mesenterium and mesenterial lymph nodes were obtained from a patient having a colonic adenocarcinoma. A normal lymph node adjacent to the salivary gland was enucleated because of its abnormal size. The tonsils from two patients and the two appendixes had no diagnostic changes. Two lymphangiomyomas and three cystic lymphangiomas were studied with similar results.

For human tissues, which were routine samples fixed with 10% formalin for histopathological diagnosis, the normal *in situ* protocol gave just backround, whereas microwave treatment instead of proteinaase K enabled specific hybridization [18, 19].

In the mesenterium, lung and appendix lymphatic endothelia (lv) gave FLT4 signals, while veins (v), arteries (a) and capillaries (c) were negative (Fig. 6A-D and data not shown). To study whether FLT4 is expressed in the HEVs, the tonsils were studied. Indeed, in the tonsils, FLT4 specific autoradiographic grains were detected in some HEVs (E, F).

EXAMPLE 6

Analysis of FLT4 mRNA in normal and metastatic lymph node and in lymphangioma

A portion of a human mesenterial lymph node (see Example 5) was analysed for FLT4 expression. The results are shown in Figure 7.

FLT4 is expressed in the lymphatic sinuses (ls) and afferent and efferent lymphatic vessels (data not shown). The same pattern is seen in a lymph node containing adenocarcinoma metastases (C, D). Some HEVs in both normal and metastatic lymph node were also positive. In panel E, FLT4 expression is shown in a cystic lymphangioma (compare with the hematoxylin-eosin stained section in F). Notably, the specificity of FLT4 to lymphatic endothelia is evident from the comparison with the *in situ* signals for von Willebrandt factor in all blood vessels (F).

EXAMPLE 7

Localization of FLT4 in fetal endothelial cells

An FLT4 cDNA fragment encoding the 40 carboxy terminal amino acids of the short form was cloned as a 657 bp *Eco*RI-fragment into the pGEX-1lT bacterial expression vector (Pharmacia) in frame with the glutatione-S-transferase coding region. The resulting GST-FLT4 fusion protein was produced in *E.coli* and purified by affinity chromatography using a glutathione-Sepharose 4B column. The purified protein was lyophilized, disolved in PBS, mixed with Freund's adjuvant and used for immunization of rabbits. Antisera were used after the fourth booster immunization.

Tissues from 17 and 20-week-old human fetuses were obtained from legal abortions induced with prostaglandins. The study was approved by the Ethical Committee of the Helsinki University Central Hospital. The

gestational age was estimated from the fetal foot length. The fetal tissues were embedded in Tissue-Tek (Miles), frozen immediately and stored at -70 °C.

Anti-FLT4 antiserum was cross-absorbed to a GST-Sepharose column to remove anti-GST-antibodies and then purified by GST-FLT4 affinity chromatography. Several 6 µm-thick cryostat sections of the tissues were fixed with acetone and treated with 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. After washing, the sections were incubated with 5% normal swine serum. Sections were then incubated with antibodies against FLT4, washed and bound antibodies were detected with peroxidase-conjugated swine anti-rabbit IgG followed by staining for peroxidase activity using 0.2% 3,3-diaminobenzidine (Amersham) as a substrate. The sections were counterstained in Meyer's hematoxylin.

Anti-FLT4 immunoperoxidase staining of human fetal mesenterium showed FLT4 protein in the endothelium of several vessels (Fig. 4A), while control stainings with antigen-blocked anti-FLT4 antibodies (B) and preimmune sera (C) were negative. For comparison, Figure 4D shows results of staining using an antiserum against the factor VIII-related antigen, which is specific for vascular endothelial cells.

EXAMPLE 8

Production of monoclonal antibodies against FLT4

Fusion I:

Four months old Balb/c male mice were immunized by intraperitoneal injection of the recombinantly produced FLT4 protein (see Example 7) in concentrated medium (150 µg/mouse), emulsified with Freund's complete adjuvant. Booster injections of 150 µg were given at three to four week intervals and a final booster (10 µg FLT4 in PBS administered intraperitoneally) was given after another three-week interval. Four days after the final booster dose, the mice were sacrified and mouse splenic lymphoid cells were fused with SP 2/0 plasmacytoma cells at a 2:1 ratio, respectively.

The fused cells were harvested in 96-well culture plates (NUNC) in Ex-Cell 320 medium (SERALAB) containing 20% fetal calf serum and HAT supplement (hypoxanthine-aminopterin-thymidine; GIBCO, 043-01060H; diluted 50-fold). Cells were cultured at +37°C, ina 5% CO_2 atmosphere. After 10 days, HAT-supplemented medium was changed to HT-supplemented cell culture medium (GIBCO; 043-01065H, diluted 50-fold). HT medium is identical to HAT medium, but lacks aminopterin.

In three weeks specific antibody production was determined by the antigen-specific immunofluorometric assay, IFMA, described in Example 10 The master clones were cloned by limited dilutions as described by Staszewski et al., Yale Journal of Biology and Medicine, 57:865-868 (1984). Positive clones were expanded onto 24-well tissue culture plates (NUNC), recloned, and re-tested by the same method. Positive clones were tested by fluorescence-activated cell sorting (FACS).

The stable clones secreted immunoglobulins belonging to the IgG1 class, except one, which produced Ig probably belonging to class IgA. The subclass of monoclonal antibody was determined using rat monoclonal antibody to mouse subclass as biotin conjugate (SEROTEC) in IFMA.

Balb/c mice were used to produce monoclonal antibodies in ascites fluid. The hybridomas described above were intraperitoneally injected into mice after pretreatment of the animals with pristane (2,6,10,14-tetramethylpentadecan 98%, ALDRICH-CHEMIE D7924 Steinheim, Cat.No. T 2,280-2). 0.5 ml of pristane (i.v.) was injected about two weeks prior to the hybridoma cells. The amount of cells injected were approximately 7.5 to 9 x 10^6 per mouse. Ascites was collected 10 to 14 days after injection of the hybridomas.

Fusion II:

Two months old Balb/c mice (female) were immunized by intraperitoneal injection of the recombinantly produced FLT-4 protein (see Example 7) (20 µg/mouse), emulsified with Freund's complete adjuvant. Booster injections of 20 µg were given at three to four week intervals and a final booster (10 µg FLT-4 in PBS administered i.v.) was given after another three-week interval. Four days after the final booster dose, the mice were

sacrified and mouse splenic lymphoid cells were fused with SP 2/0 plasmacytoma cells at a 2:1 ratio, respectively.

The fused cells were harvested in 96-well culture plates (FALCON) in OptiMEM 1 (with Glutamax 1, 51985-026, GIBCO BRL) medium containing 20 % fetal calf serum and HAT supplement (hypoxanthine-aminopterin-thymidine; GIBCO BRL 21060-017; diluted 1:50 fold). Cells were cultured at +37 °C, in a 5% CO2 atmosphere. After 10 days, HAT-supplemented medium was changed to HT-supplemented cell culture medium (GIBCO BRL; 41065-012, diluted 1:50-fold). HT-medium is identical to HAT-medium,but lacks aminopterin.

In three weeks specific antibody production was determined by the antigen-specific ImmunoFluoroMetric Assay (IFMA) described in Example 9. The master clones were cloned by limited dilutions as described by Staszewski et al., Yale Journal of Biology and Medicine, 57:865-868 (1984). Positive clones were expanded onto 24-well tissue culture plates (FALCON), recloned, and re-tested by the same method. Positive clones were tested by fluorescence-activated cell sorting (FACS).

The 2E11 and 6B2 clones secreted immunoglobulins belonging to the IgG_1 class, 2B12 clones produced Ig belonging to subclass IgM . The mouse subclass IgG_1 was determined using rat monoclonal antibody against mouse subclass heavy chain as biotin conjugate (SEROTEC) in IFMA and the mouse subclass IgM was determined with Mouse Monoclonal Antibody Isotyping Kit (Dipstick Format) (19663-012, Life Technologies Inc.).

EXAMPLE 9

Specificity of monoclonal antibodies against FLT4

Fusion I antibodies:

The extracellular domain of FLT4 described in Example 7, was labelled according to Mukkala et al., in *Anal.Biochem.* 176(2):319-325, 1989 , with the following modification: a 250 times molar excess of isothiocyanate DTTA-Eu (N1 chelate, WALLAC, Finland) was added to the FLT4 solution (0.5 mg/ml in PBS) and the pH was adjusted to about 9 by adding 0.5 mol/L

sodium carbonate buffer, pH 9.8. The labelling was performed overnight at +4°C. Unbound label was removed using PD-10 (PHARMACIA, Sweden) with TSA buffer (50 mmol/L Tris-HCl, pH 7.8 containing 0.15 mol/l NaCl) as eluent.

After purification, 1 mg/ml bovine serum albumin (BSA) was added to the labelled FLT4 and the label was stored at +4°C. The number of europium ions incorporated per FLT4 molecule was 1.9, as determined by measuring the fluorescence in a ratio to that of known $EuCl_3$ standards (Hemmilä et al., *Anal.Biochem.*, 137:335-343, 1984).

The antibodies produced in Example 8, were screened using a Sandwich type immunofluorometric assay using microtitration strip wells (NUNC, polysorb) coated with rabbid anti-mouse Ig (Z 259, DAKOPATTS). The precoated wells were washed once by Platewash 1296-024 (WALLAC) with DELFIA wash solution. The DELFIA assay buffer was used as a dilution buffer for cell culture supernatants and for serum of the spleenectomized mouse (at dilutions between 1:1000 to 1: 100 000) used as positive control in the preliminary screening assay.

An overnight incubation at +4°C (or alternatively for 2 hours at room temperature) was begun by shaking on a Plateshake shaker (1296-001, WALLAC) for 5 min followed by washing four times with wash solution as described above.

The europium-labelled FLT4 was added at a dilution of 1:500 in 100 µl of the assay buffer. After 5 min on a Plateshake shaker and one hour incubation at RT the strips were washed as described above.

Enhancement solution (DELFIA) was added at 200 µl/well. The plates were then shaken for 5 min an a Plateshake shaker and the intensity of fluorescence was measured by ARCUS-1230 (WALLAC) for 10-15 min. (Lövgren et al., In: Collins W.P. (Ed) Alternative Immunoassays, John Wiley & Sons Ltd, 1985; pp. 203-216).

The resulting.monoclonal antibodies against FLT4 and corresponding FACS results are summarized in Table 2.

TABLE 2

Mab clones	LTR%[a)]	NEO%[b)]	DELFIA-counts
1B1	67,3	1	20625
1B1D11	75	1,2	19694
1B1F8	76,1	1,4	18580
4F6	69,9	1,2	23229
4F6B8G12	75	0,3	24374
4F6B8H11	75,9	0,3	28281
4F6B8E12	74,8	0,4	27097
4F6B8G10	75,3	0,4	26063
9D9	45,1	0,75	17316
9D9D10	71,7	2,3	18230
9D9F9	73	1,8	11904
9D9G6	74,3	2,9	16743
9D9G7	70,7	1,3	17009
10E4	24,2	1,4	39202
10E4B10E12	32,3	0,3	42490
10E4B10G10	36,5	0, 3	54815
10E4B10F12	45,6	0,4	43909
10E4B10G12	45,7	0,5	35576
11G2	30,2	1,6	11304
11G2D12	74,4	1,5	14660
11G2G9	74,2	0,9	10283
11G2H7	74,4	2,1	25382

a) FACS results wiht LTR transfected cells
b) FACS results wiht NEO cells (control)

One clone, designated anti-FLT4 9D9F9 was found to stably secrete monoclonal antibody which was determined to be of immunoglobulin class IgG1 by IFMA. Hybridoma 9d9f9 was deposited with the German Collection of Microorganisms and Cell Cultures, Department of Human and Animal Cell Cultures and Viruses, Mascheroder Weg 1b, 3300 Braunschweig, Germany, March 23, 1995, and given accession No. ACC2210.

Fusion II antibodies:

The extracellular domain of FLT-4 described in Example 7, was labelled according to Mukkala et al., in Anal.Biochem. 176(2): 319-325, 1989, with the following modification: a 250 times molar excess of isothiocyanate DTTA-Eu (N1 chelate, Wallac, Finland) was added to the FLT-4 solution (0.5 mg/ml in PBS) and the pH was adjusted to about 9 by adding 0.5 mol/L sodium carbonate buffer, pH 9.8. The labelling was performed overnight at +4°C. Unbound label was removed using PD-10 (PHARMACIA) with TSA buffer (50 mmol/L Tris-HCl, pH 7.8 containing 0.15 mol/L NaCl) as eluent.

After purification, 1 mg/ml bovine serum albumin (BSA) was added to the labelled FLT-4 and the label was stored at +4°C. The number of europium ions incorporated per FLT-4 molecule was 1.9, as determined by measuring the fluorescence in a ratio to that of known EuCl3 standards (Hemmil et al., Anal.Biochem., 137: 335-343, 1984).

The antibodies produced in Example 8, were screened using a FLT-4 specific IFMA using microtitration wells (Nunc, Polysorb) coated with rabbit antimouse Ig (Z 259, DAKO). The precoated wells were washed once with wash solution (Wallac) by using DELFIA Plate wash.

The DELFIA assay buffer was used as dilution buffer for cell culture supernatants (dilution 1:2 in preliminary screening) and for serum of the splenectomized mouse (dilutions 1:1 000 to 1:100 000) which was used as positive control. As standard the purified antiFLT-4 9D9F9 (mouse subclass IgG1) was used at concentrations between 1.0 ng/ml and 250 ng/ml. Samples were first shaken at room temperature for five minutes on Plate shake (Wallac) and then incubated approx. 18 hours at +4°C. The frames were first washed four times, then the Eu-labelled FLT-4 (1:2000, in 100 µl assay buffer) was added and finally the frames were incubated for one hour at room temperature. After washing as described the enhancement solution (200 µl/well, Wallac) was added and the frames were shaken for 5 minutes on Plate shake. The intensity of fluorescence was measured by ARCUS-1230 (Wallac).

The resulting monoclonal antibodies against FLT-4 and corresponding results are summarized in Table 3.

A standard curve for quantitation of antiFLT-4 antibodies was made by using affinity purified antiFLT-4 9D9F9. The linear range reached from 1.0 ng/ml to 250 ng/ml.

Cell lysate of NIH 3T3 cells cotranfected with pLTRFLT4 construct expressing full-length FLT4 on the surface was electrophoresed in 6.5% SDS-PAGE, proteins were transfered onto nitrocellulose nitrate membrane (0.45 µm, SCHLEICHER & SCHUELL) and immunoblotted with Mab cell culture supernatants (1:10, 50 mmol/L TRIS - 40 mmol/L glycine buffer containing methanol 4%, SDS 0.04%). The specificity of Mab was detected using incubation with HRP-conjugated rabbit antimouse Ig (P 161, DAKO, diluted 1:1000 in 20 mmol/L TRIS buffer pH 7.5 containing 150 mmol/L saline, 5% milk powder) and ECL (Enhanced chemiluminescence, AMERSHAM).

TABLE 3.

Mab clones	LTR %a)	NEOb)	approx.Mab prod. ng/ml/106 cells	WB
2B12E10	39.5	6.0	440	+
2E11D11	44.6	8.8	110	+
2E11F9	49.5	4.5	100	+
2E11F12	46.0	4.1	180	+
2E11G8	41.2	7.8	160	+
6B2E12	NF	NF	1390	+
6B2F8	NF	NF	470	+
6B2G6	NF	NF	630	+
6B2H5	NF	NF	740	+
6B2H8	NF	NF	1800	+

a) FACS results with LTR transfected cells
b) FACS results with NEO cells (control)
NF not functioning in FACS
c) quantitation of Mab production based on affinity purified antiFLT 9D9F9 antibody used as standard

As is evident from the foregoing, antibodies according to the present invention are useful in the diagnosis and identification of lymphatic vessels, lymphatic endothelial cells, high endothelial venules, lymphangiomas, metastatic lymph nodes and other disease states of the lymphatic system, the detection and monitoring of metastatic spread, in the stimulation and inhibition of endothelial cells of lymphatic vessels and high endothelial venules, in the introduction of molecules selectively into endothelial cells and in the imaging of lymphatic vessels and their disease states. Other uses of the presently-claimed subject matter are apparent to the skilled artisan.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, line 13 - 17.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b
D-38124 Braunschweig
Germany

Date of deposit	Accession Number
1995-03-23	DSM ACC2210

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

As regards the respective Patent Offices of the respective designated states, the applicant requests that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

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  1108
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  1588
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  1636
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    525                 530                 535

GTG GGC CAG GAT GAG CGG CTC ATC TAC TTC TAT GTG ACC ACC ATC CCC
  1684
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540                 545                 550                 555

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  1732
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  1780
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CTG CGC TGG TAC CGC CTC AAC CTG TCC ACG CTG CAC GAT GCG CAC GGG
  1828
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        590                 595                 600
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AAC CCG CTT CTG CTC GAC TGC AAG AAC GTG CAT CTG TTC GCC ACC CCT
  1876
Asn Pro Leu Leu Leu Asp Cys Lys Asn Val His Leu Phe Ala Thr Pro
    605                 610                 615

CTG GCC GCC AGC CTG GAG GAG GTG GCA CCT GGG GCG CGC CAC GCC ACG
  1924
Leu Ala Ala Ser Leu Glu Glu Val Ala Pro Gly Ala Arg His Ala Thr
620                 625                 630                 635

CTC AGC CTG AGT ATC CCC CGC GTC GCG CCC GAG CAC GAG GGC CAC TAT
  1972
Leu Ser Leu Ser Ile Pro Arg Val Ala Pro Glu His Glu Gly His Tyr
                640                 645                 650

GTG TGC GAA GTG CAA GAC CGG CGC AGC CAT GAC AAG CAC TGC CAC AAG
  2020
Val Cys Glu Val Gln Asp Arg Arg Ser His Asp Lys His Cys His Lys
            655                 660                 665

AAG TAC CTG TCG GTG CAG GCC CTG GAA GCC CCT CGG CTC ACG CAG AAC
  2068
Lys Tyr Leu Ser Val Gln Ala Leu Glu Ala Pro Arg Leu Thr Gln Asn
        670                 675                 680

TTG ACC GAC CTC CTG GTG AAC GTG AGC GAC TCG CTG GAG ATG CAG TGC
  2116

Leu Thr Asp Leu Leu Val Asn Val Ser Asp Ser Leu Glu Met Gln Cys
    685                 690                 695

TTG GTG GCC GGA GCG CAC GCG CCC AGC ATC GTG TGG TAC AAA GAC GAG
  2164
Leu Val Ala Gly Ala His Ala Pro Ser Ile Val Trp Tyr Lys Asp Glu
700                 705                 710                 715

AGG CTG CTG GAG GAA AAG TCT GGA GTC GAC TTG GCG GAC TCC AAC CAG
  2212
Arg Leu Leu Glu Glu Lys Ser Gly Val Asp Leu Ala Asp Ser Asn Gln
                720                 725                 730

AAG CTG AGC ATC CAG CGC GTG CGC GAG GAG GAT GCG GGA CGC TAT CTG
  2260
Lys Leu Ser Ile Gln Arg Val Arg Glu Glu Asp Ala Gly Arg Tyr Leu
            735                 740                 745

TGC AGC GTG TGC AAC GCC AAG GGC TGC GTC AAC TCC TCC GCC AGC GTG
  2308
Cys Ser Val Cys Asn Ala Lys Gly Cys Val Asn Ser Ser Ala Ser Val
        750                 755                 760

GCC GTG GAA GGC TCC GAG GAT AAG GGC AGC ATG GAG ATC GTG ATC CTT
  2356
Ala Val Glu Gly Ser Glu Asp Lys Gly Ser Met Glu Ile Val Ile Leu
    765                 770                 775

GTC GGT ACC GGC GTC ATC GCT GTC TTC TTC TGG GTC CTC CTC CTC CTC
  2404
Val Gly Thr Gly Val Ile Ala Val Phe Phe Trp Val Leu Leu Leu Leu
780                 785                 790                 795

ATC TTC TGT AAC ATG AGG AGG CCG GCC CAC GCA GAC ATC AAG ACG GGC
  2452
Ile Phe Cys Asn Met Arg Arg Pro Ala His Ala Asp Ile Lys Thr Gly
                800                 805                 810
```

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  2500
Tyr Leu Ser Ile Ile Met Asp Pro Gly Glu Val Pro Leu Glu Glu Gln
          815                 820                 825

TGC GAA TAC CTG TCC TAC GAT GCC AGC CAG TGG GAA TTC CCC CGA GAG
  2548
Cys Glu Tyr Leu Ser Tyr Asp Ala Ser Gln Trp Glu Phe Pro Arg Glu
      830                 835                 840

CGG CTG CAC CTG GGG AGA GTG CTC GGC TAC GGC GCC TTC GGG AAG GTG
  2596
Arg Leu His Leu Gly Arg Val Leu Gly Tyr Gly Ala Phe Gly Lys Val
  845                 850                 855

GTG GAA GCC TCC GCT TTC GGC ATC CAC AAG GGC AGC AGC TGT GAC ACC
  2644
Val Glu Ala Ser Ala Phe Gly Ile His Lys Gly Ser Ser Cys Asp Thr
860                 865                 870                 875

GTG GCC GTG AAA ATG CTG AAA GAG GGC GCC ACG GCC AGC GAG CAC CGC
  2692
Val Ala Val Lys Met Leu Lys Glu Gly Ala Thr Ala Ser Glu His Arg
              880                 885                 890

GCG CTG ATG TCG GAG CTC AAG ATC CTC ATT CAC ATC GGC AAC CAC CTC
  2740
Ala Leu Met Ser Glu Leu Lys Ile Leu Ile His Ile Gly Asn His Leu
          895                 900                 905

AAC GTG GTC AAC CTC CTC GGG GCG TGC ACC AAG CCG CAG GGC CCC CTC
  2788
Asn Val Val Asn Leu Leu Gly Ala Cys Thr Lys Pro Gln Gly Pro Leu
      910                 915                 920

ATG GTG ATC GTG GAG TTC TGC AAG TAC GGC AAC CTC TCC AAC TTC CTG
  2836
Met Val Ile Val Glu Phe Cys Lys Tyr Gly Asn Leu Ser Asn Phe Leu
  925                 930                 935

CGC GCC AAG CGG GAC GCC TTC AGC CCC TGC GCG GAG AAG TCT CCC GAG
  2884
Arg Ala Lys Arg Asp Ala Phe Ser Pro Cys Ala Glu Lys Ser Pro Glu
940                 945                 950                 955

CAG CGC GGA CGC TTC CGC GCC ATG GTG GAG CTC GCC AGG CTG GAT CGG
  2932
Gln Arg Gly Arg Phe Arg Ala Met Val Glu Leu Ala Arg Leu Asp Arg
              960                 965                 970

AGG CGG CCG GGG AGC AGC GAC AGG GTC CTC TTC GCG CGG TTC TCG AAG
  2980
Arg Arg Pro Gly Ser Ser Asp Arg Val Leu Phe Ala Arg Phe Ser Lys
          975                 980                 985

ACC GAG GGC GGA GCG AGG CGG GCT TCT CCA GAC CAA GAA GCT GAG GAC
  3028
Thr Glu Gly Gly Ala Arg Arg Ala Ser Pro Asp Gln Glu Ala Glu Asp
      990                 995                 1000

CTG TGG CTG AGC CCG CTG ACC ATG GAA GAT CTT GTC TGC TAC AGC TTC
  3076
Leu Trp Leu Ser Pro Leu Thr Met Glu Asp Leu Val Cys Tyr Ser Phe
  1005                1010                1015
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CAG GTG GCC AGA GGG ATG GAG TTC CTG GCT TCC CGA AAG TGC ATC CAC
  3124
Gln Val Ala Arg Gly Met Glu Phe Leu Ala Ser Arg Lys Cys Ile His
1020                1025                1030                1035

AGA GAC CTG GCT GCT CGG AAC ATT CTG CTG TCG GAA AGC GAC GTG GTG
  3172
Arg Asp Leu Ala Ala Arg Asn Ile Leu Leu Ser Glu Ser Asp Val Val
                1040                1045                1050

AAG ATC TGT GAC TTT GGC CTT GCC CGG GAC ATC TAC AAA GAC CCT GAC
  3220
Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Tyr Lys Asp Pro Asp
            1055                1060                1065

TAC GTC CGC AAG GGC AGT GCC CGG CTG CCC CTG AAG TGG ATG GCC CCT
  3268
Tyr Val Arg Lys Gly Ser Ala Arg Leu Pro Leu Lys Trp Met Ala Pro
        1070                1075                1080

GAA AGC ATC TTC GAC AAG GTG TAC ACC ACG CAG AGT GAC GTG TGG TCC
  3316
Glu Ser Ile Phe Asp Lys Val Tyr Thr Thr Gln Ser Asp Val Trp Ser
    1085                1090                1095

TTT GGG GTG CTT CTC TGG GAG ATC TTC TCT CTG GGG GCC TCC CCG TAC
  3364
Phe Gly Val Leu Leu Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr
1100                1105                1110                1115

CCT GGG GTG CAG ATC AAT GAG GAG TTC TGC CAG CGG CTG AGA GAC GGC
  3412
Pro Gly Val Gln Ile Asn Glu Glu Phe Cys Gln Arg Leu Arg Asp Gly
                1120                1125                1130

ACA AGG ATG AGG GCC CCG GAG CTG GCC ACT CCC GCC ATA CGC CGC ATC
  3460
Thr Arg Met Arg Ala Pro Glu Leu Ala Thr Pro Ala Ile Arg Arg Ile
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ATG CTG AAC TGC TGG TCC GGA GAC CCC AAG GCG AGA CCT GCA TTC TCG
  3508
Met Leu Asn Cys Trp Ser Gly Asp Pro Lys Ala Arg Pro Ala Phe Ser
        1150                1155                1160

GAG CTG GTG GAG ATC CTG GGG GAC CTG CTC CAG GGC AGG GGC CTG CAA
  3556
Glu Leu Val Glu Ile Leu Gly Asp Leu Leu Gln Gly Arg Gly Leu Gln
    1165                1170                1175

GAG GAA GAG GAG GTC TGC ATG GCC CCG CGC AGC TCT CAG AGC TCA GAA
  3604
Glu Glu Glu Glu Val Cys Met Ala Pro Arg Ser Ser Gln Ser Ser Glu
1180                1185                1190                1195

GAG GGC AGC TTC TCG CAG GTG TCC ACC ATG GCC CTA CAC ATC GCC CAG
  3652
Glu Gly Ser Phe Ser Gln Val Ser Thr Met Ala Leu His Ile Ala Gln
                1200                1205                1210

GCT GAC GCT GAG GAC AGC CCG CCA AGC CTG CAG CGC CAC AGC CTG GCC
  3700
Ala Asp Ala Glu Asp Ser Pro Pro Ser Leu Gln Arg His Ser Leu Ala
            1215                1220                1225
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  3748
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GCT GAG ACC CGT GGT TCC TCC AGG ATG AAG ACA TTT GAG GAA TTC CCC
  3796
Ala Glu Thr Arg Gly Ser Ser Arg Met Lys Thr Phe Glu Glu Phe Pro
    1245                1250                1255

ATG ACC CCA ACG ACC TAC AAA GGC TCT GTG GAC AAC CAG ACA GAC AGT
  3844
Met Thr Pro Thr Thr Tyr Lys Gly Ser Val Asp Asn Gln Thr Asp Ser
1260                1265                1270                1275

GGG ATG GTG CTG GCC TCG GAG GAG TTT GAG CAG ATA GAG AGC AGG CAT
  3892
Gly Met Val Leu Ala Ser Glu Glu Phe Glu Gln Ile Glu Ser Arg His
                1280                1285                1290

AGA CAA GAA AGC GGC TTC AGG TAGCTGAAGC AGAGAGAGAG AAGGCAGCAT
  3943
Arg Gln Glu Ser Gly Phe Arg
            1295

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  4003

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  4063

GAAAGTGGAC AAGGAGTGTG ACCACTGAAG CACCACAGGG AAGGGGTTAG GCCTCCGGAT
  4123

GACTGCGGGC AGGCCTGGAT AATATCCAGC CTCCCACAAG AAGCTGGTGG AGCAGAGTGT
  4183

TCCCTGACTC CT
  4195
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CLAIMS

1. An antibody directed against an FLT4 receptor tyrosine kinase.

2. A polyclonal antibody directed against an FLT4 receptor tyrosine kinase.

3. A monoclonal antibody directed against an FLT4 receptor tyrosine kinase.

4. The monoclonal antibody according to claim 3, wherein said antibody is anti-FLT4 monoclonal antibody 9D9F9.

5. A hybridoma cell line producing the monoclonal antibody according to claim 4.

6. The hybridoma cell line according to claim 5, wherein said hybridoma cell line is deposited as DSM ACC 2210.

7. A monoclonal antibody produced by the hybridoma cell line according to claim 6.

8. A detectably-labelled antibody according to claim 1.

9. A nucleic acid probe specific for DNA encoding human FLT4 comprising the nucleic acid sequence of SEQ ID NO. 1 or fragments thereof which specifically bind/hybridize to DNA encoding human FLT4.

10. A method for detecting FLT4 in a biological sample; comprising the steps of

a) exposing a sample suspected of containing FLT4 to a detectably-labelled probe according to claim 6;

b) washing the sample; and

c) detecting the prescence of said detectably-labelled probe.

11. A method for detecting FLT4 in a biological sample, comprising the steps of

a) exposing a sample suspected of containing FLT4 to a detectably-labelled anti-FLT4 antibody;

b) washing the sample; and

c) detecting the presence of said detectably-labelled anti-FLT4 antibody in said sample.

12. A method for imaging lympahtic vessels in a tissue sample, comprising the steps of:

a) applying a detectably labelled anti-FLT4 antibody to a site suspected of containing lymphatic vessels; and

b) detecting said detectably-labelled anti-FLT4 antibody bound to said tissue.

13. A method for diagnosing diseases characterized by changes in lymphatic vessels and HEVs, comprising the steps of:

a) obtaining a tissue sample from a patient suspected of having a disease characterized by changes in lymphatic cells and HEVs;

b) exposing said tissue sample to a detectably-labelled anti-FLT4 antibody;

c) washing the said tissue sample; and

d) detecting the presence of said detectably-labelled anti-FLT4 in said tissue sample.

14. A pharmaceutical composition comprising a therapeutically effective amount of an FLT4-binding compound in a pharmaceutically acceptable diluent, adjuvant or carrier, said composition being in a form suitable for injection.

15. The pharmaceutical composition according to claim 14, wherein the FLT4-binding compond comprises a detectably labelled anti-FLT4 antibody in a a pharmaceutically acceptable diluent, adjuvant or carrier, said composition being in a form suitable for injection.

16. A method for detecting lymphatic vessels and HEVs in an organism, comprising the steps of

a) injecting a pharmaceutical compostion according to claim 14 or 15, into said organism;

b) detecting the the amount of said detectably labelled FLT4-binding compound bound to sites comprising lymphatic vessels or HEVs in said organism.

17. The method according to claim 16, wherein the lymphatic tissue to be detected is lymph node tissue.

18. A method for stimulating or antagonizing the function of FTL4 in an organism comprising the step of providing a pharmaceutical compostion according to claim 14 or 15 in an amount sufficient to stimulate or block the FLT4-receptor.

19. The method according to claim 18, wherein the FLT4 receptor activity is associated with a disease selected from the group consisting of metastatic cancers, lymphomas, lymphangiomas, inflammation (chronic or acute), infections and immunological diseases.

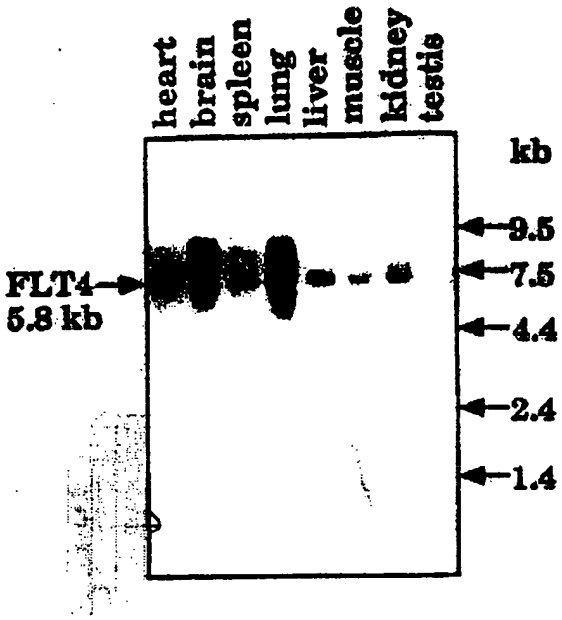
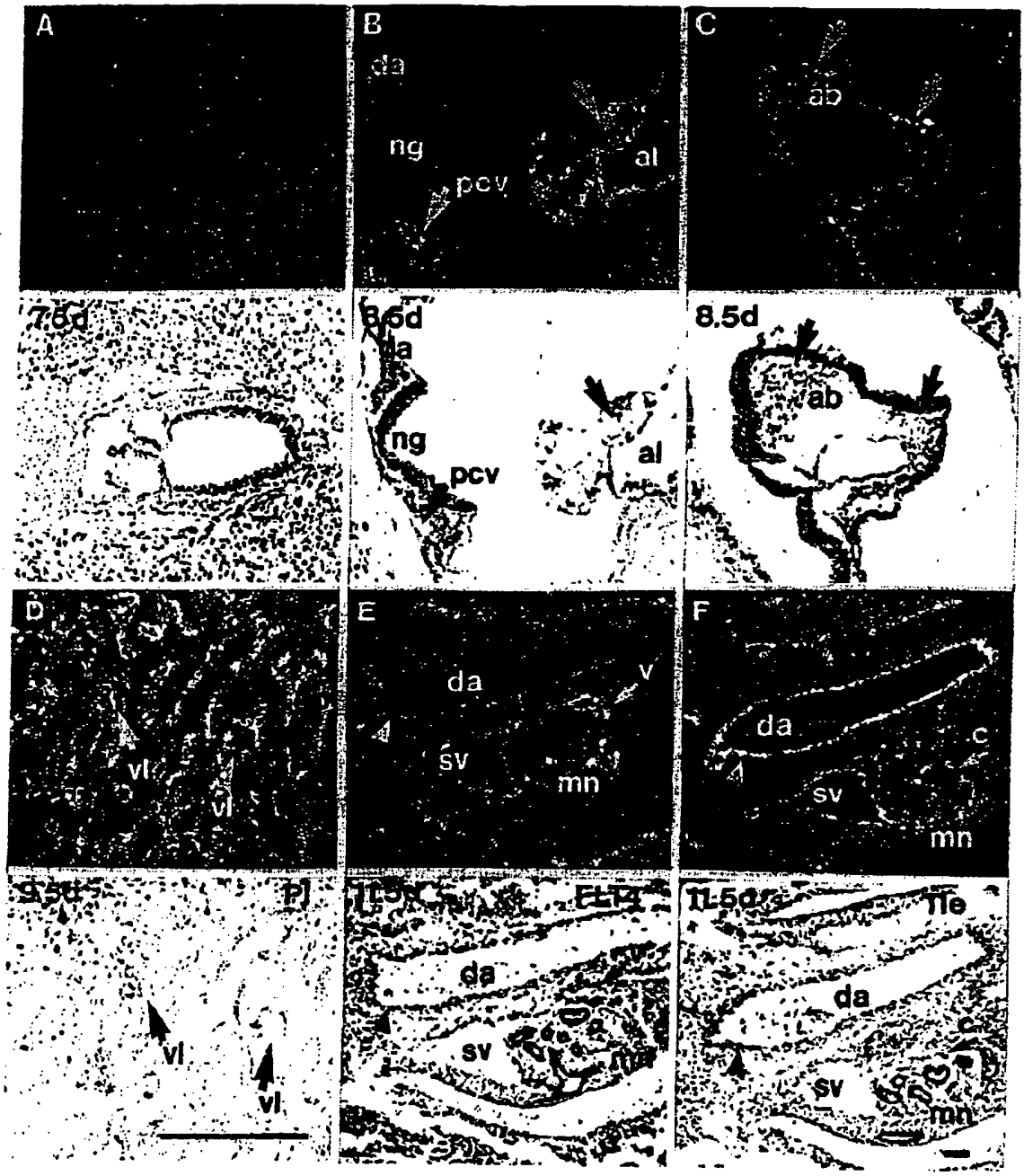

heart
brain
spleen
lung
liver
muscle
kidney
testis
kb
9.5
7.5
4.4
2.4
1.4
FLT4
5.8 kb

Fig. 1A


FLT4 probe
E8 +P
E10
E12
E14
E16
E18
NB
tRNA
265
192
FLT4
β-actin
nt

Fig. 1B


A
B
da
ng
pcv
al
C
ab
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8.5d
da
ng
pcv
al
ab
D
E
F
da
v
sv
mn
vl
vl
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11.5d
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sv
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Fig. 2

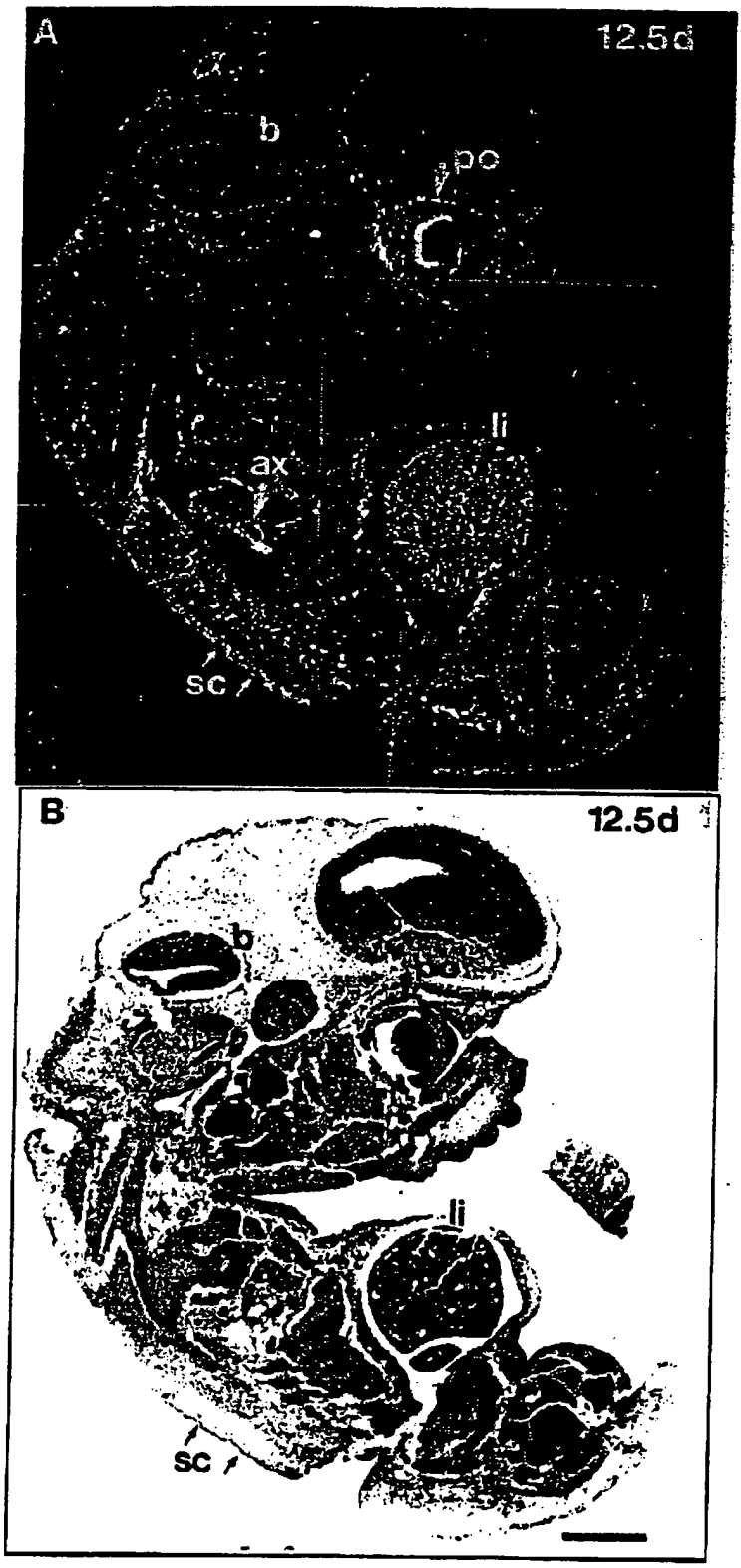

Fig. 3

SUBSTITUTE SHEET

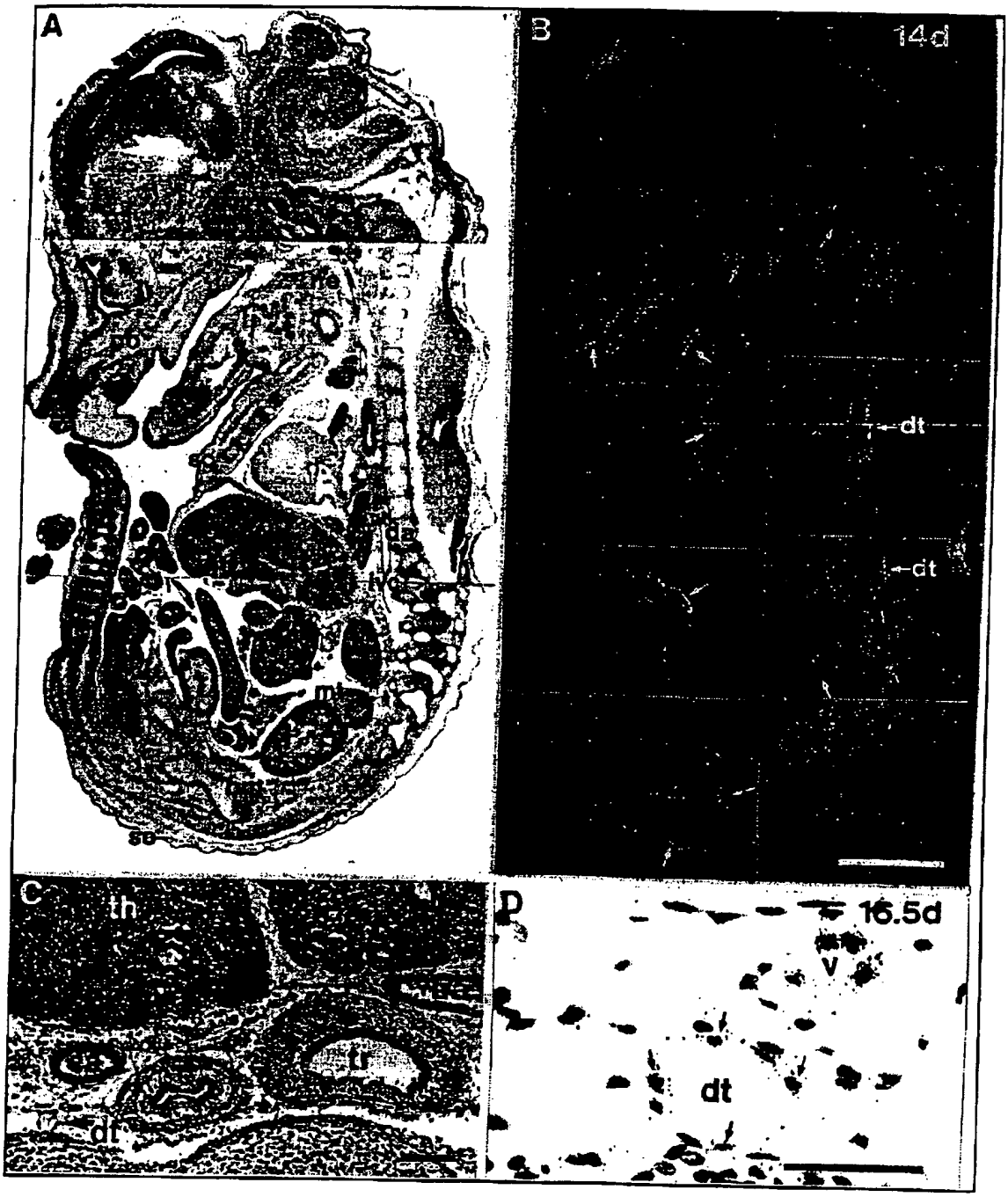

Fig. 4

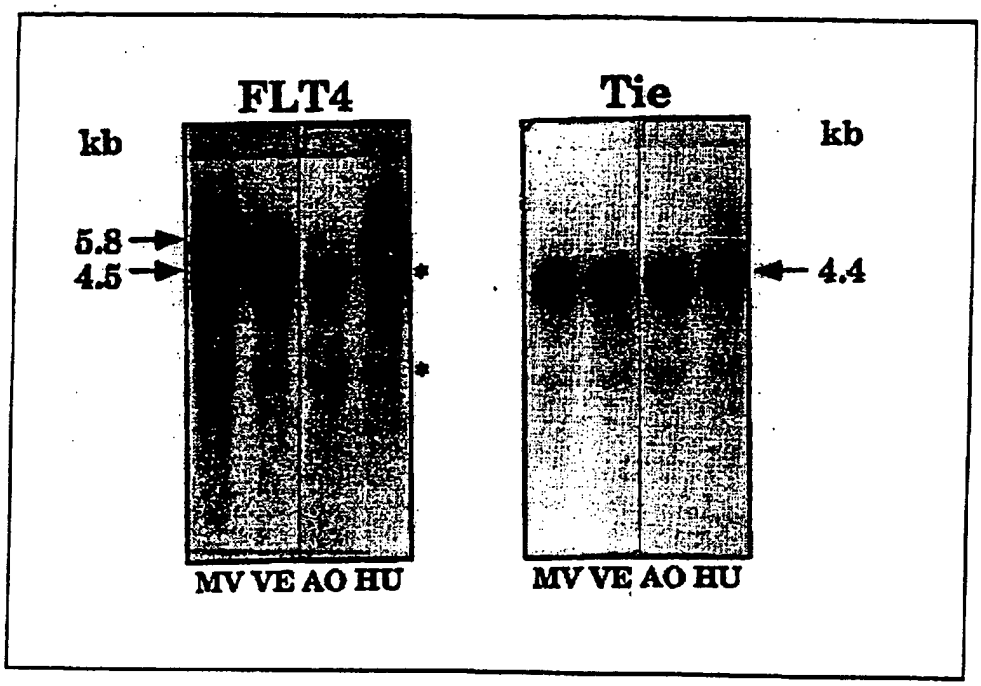

Fig. 5

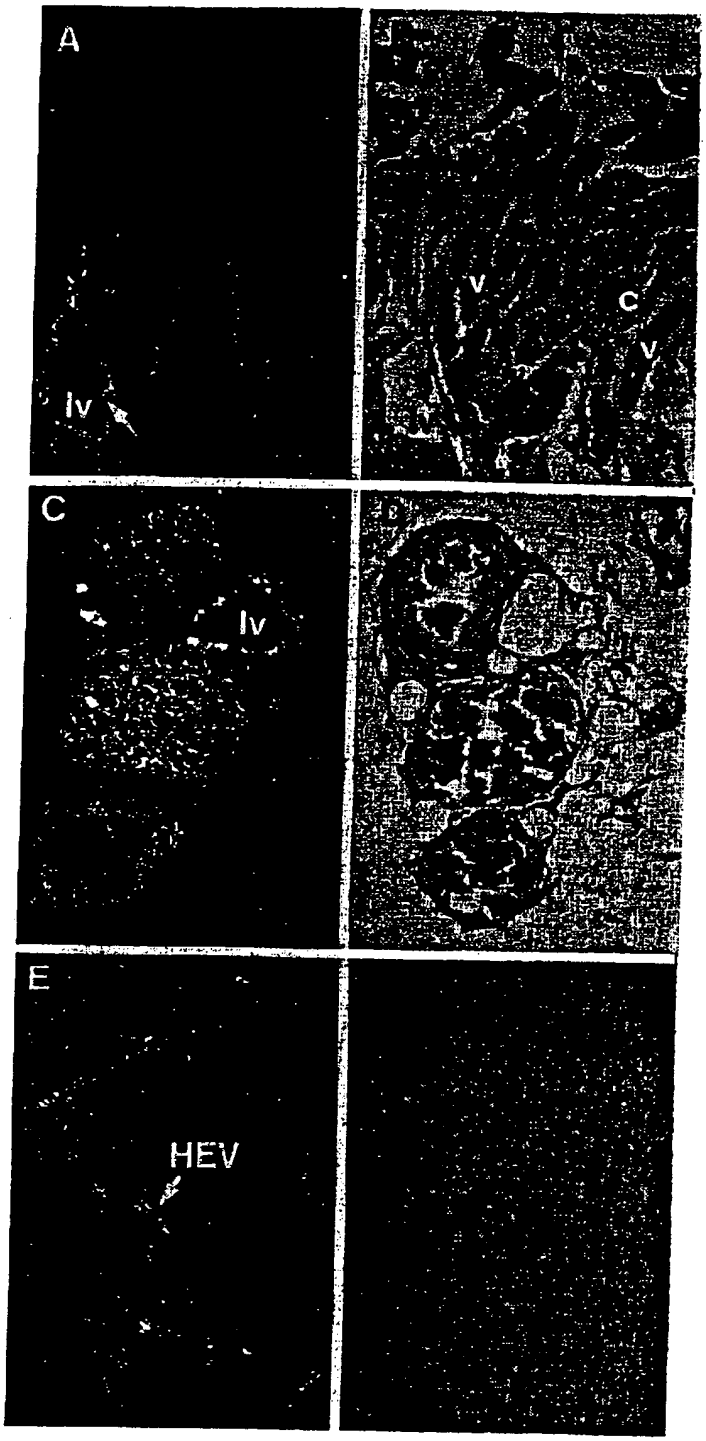

Fig. 6

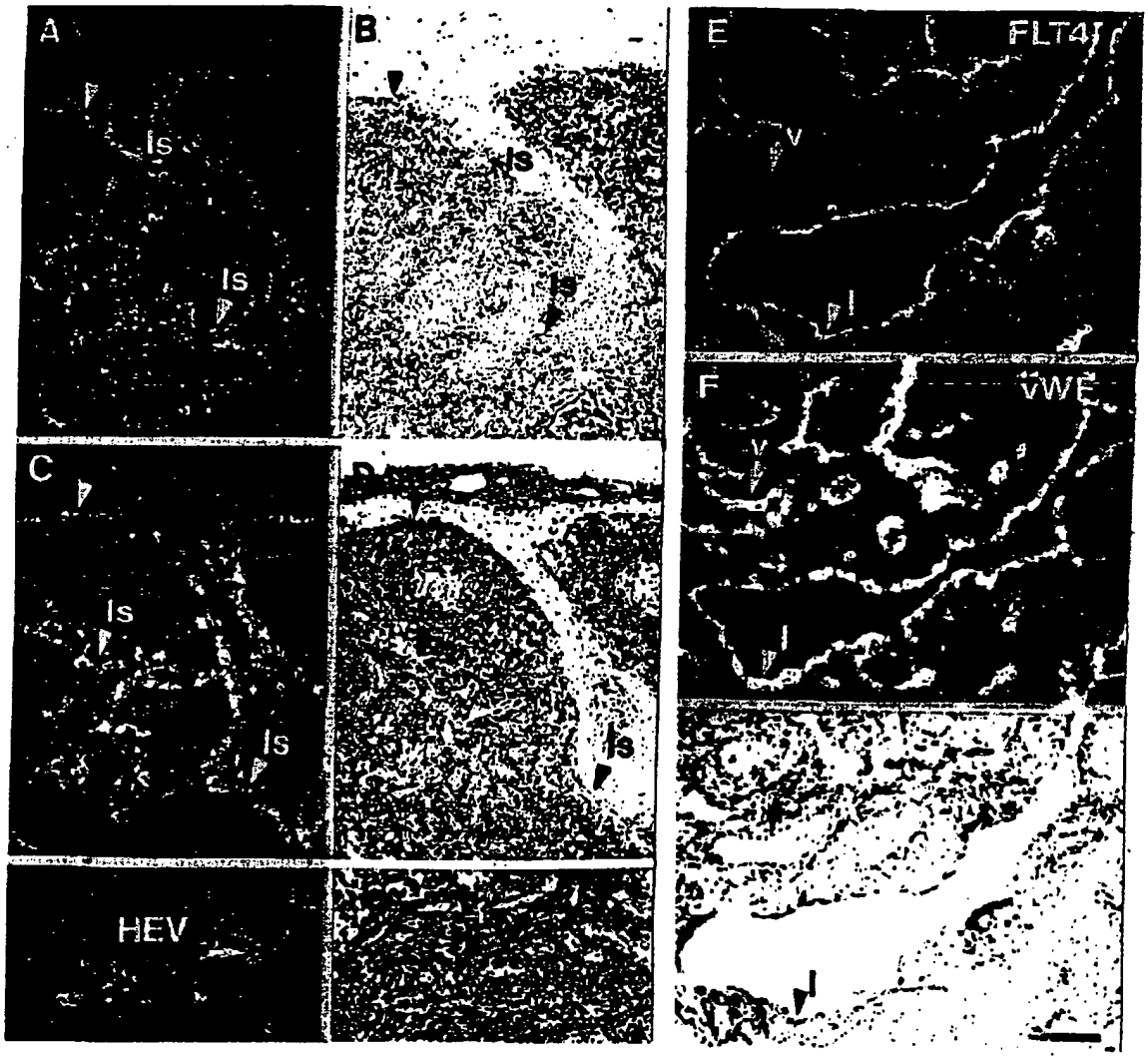

Fig. 7

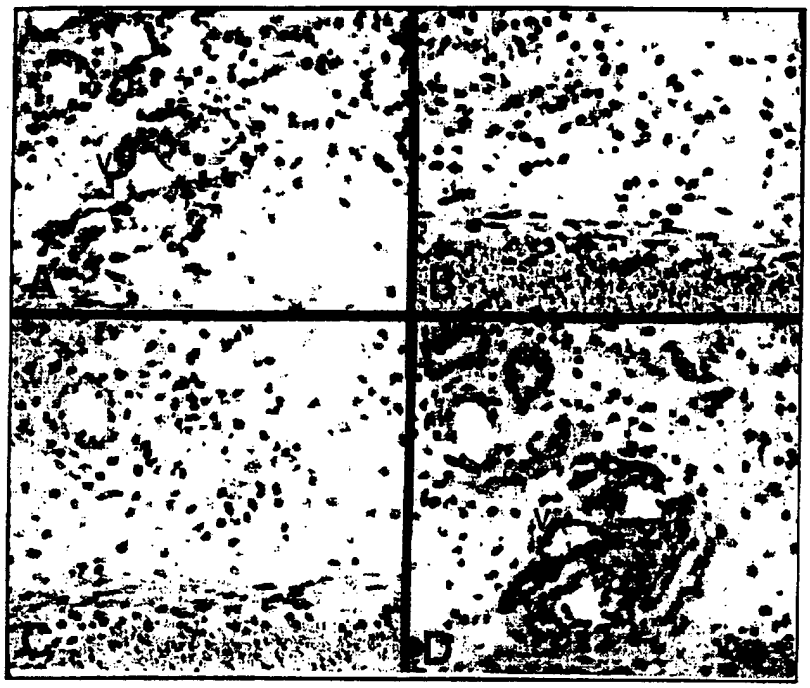

Fig. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI 95/00337

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/28 C12N5/20 C12Q1/68 G01N33/577 A61K39/395
A61K49/00 //C07K14/71

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12Q G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ONCOGENE, vol. 8, no. 11, November 1993 BASINGSTOKE, GB, pages 2931-2937, K. PAJUSOLA ET AL. 'Two human FLT4 receptor tyrosine kinase isoforms with distinct carboxy terminal tails are produced by alternative processing of primary transcripts.' see abstract see figure 1 see "material and methods" --- -/--	1,2,8-11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 October 1995	16.11.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nooij, F

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INTERNATIONAL SEARCH REPORT

International application No
PCT/FI 95/00337

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ONCOGENE, vol. 8, no. 5, May 1993 BASINGSTOKE, GB, pages 1233-1240, F. GALLAND ET AL. 'The FLT4 gene encodes a transmembrane tyrosine kinase related to the vascular endothelial growth factor receptor.' cited in the application see abstract see figures 2,4,7 see "material and methods" ---	1,2,8-11
X	CANCER RESEARCH, vol. 52, no. 20, 15 October 1992 BALTIMORE, MD, USA, pages 5738-5743, K. PAJUSOLA ET AL. 'FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines.' cited in the application see abstract see figures 1,2 ---	9,10
X	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 178, no. 6, 1 December 1993 NEW YORK, NY, USA, pages 2077-2088, A. KAIPAINEN ET AL. 'The related FLT4, FLT1, and KDR receptor tyrosine kinases show distinct expression patterns in human fetal endothelial cells.' cited in the application see abstract see page 2078, left column, line 54 - right column, line 1 ---	9,10
A	WO,A,94 10202 (GENENTECH, INC.) 11 May 1994 see examples see claims ---	1-8, 11-19
A	WO,A,93 14124 (HELSINKI UNIVERSITY HOLDING, LTD.) 22 July 1993 see claims see examples --- -/--	1,2,8-19

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INTERNATIONAL SEARCH REPORT

International application No
PCT/FI 95/00337

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 92, no. 8, 11 April 1995 WASHINGTON, DC, USA, pages 3566-3570, A. KAIPAINEN ET AL. 'Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development.' see abstract see "material and methods"	9,10

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 95/00337

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Please see annex!

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/FI 95/00337

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

REMARK: Although claims 16 and 17 are directed to a diagnostic method, and claims 18 and 19 to a medical treatment, practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internation pplicaton No
PCT/FI 95/00337

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